Serotonin Increases Interleukin-6 Synthesis in Human Vascular Smooth Muscle Cells

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Background—Interleukin-6 (IL-6) is a key molecule in chronic inflammation and has been implicated in the progression of atherosclerosis. Serotonin (5-hydroxytryptamine; 5-HT) causes vascular contraction and proliferation, but its role in atherogenesis has not been clarified. We investigated the effects of 5-HT on IL-6 synthesis in human vascular smooth muscle cells (VSMCs).

Methods and Results—IL-6 levels in the culture medium of VSMCs were determined by ELISA. IL-6 mRNA accumulation was determined by use of a Quantikine mRNA colorimetric quantification kit. NF-κB activation was tested by gel retardation assay. 5-HT induced IL-6 production by VSMCs in a time- and dose-dependent manner, with increased IL-6 mRNA accumulation and nuclear factor-κB activation. The effect of 5-HT on IL-6 production was significantly inhibited by the 5-HT 2 receptor antagonist ketanserin and the selective 5-HT 2A receptor antagonist sarpogrelate. Conversely, the 5-HT 2 receptor agonist α1-methyl-5-HT increased IL-6 production. The protein kinase C (PKC) inhibitor calphostin C, but not the protein kinase A inhibitor KT5720, suppressed 5-HT–induced IL-6 production. The effect of 5-HT was also abolished in PKC-depleted VSMCs after pretreatment with phorbol 12-myristate 13-acetate for 24 hours.

Conclusions—5-HT acts on 5-HT 2A receptors and increases IL-6 synthesis in human VSMCs at least partially through a PKC-dependent pathway. These results suggested that 5-HT may contribute to inflammatory activation of the vessels during atherogenesis. (Circulation. 2000;102:2522-2527.)

Key Words: muscle, smooth interleukins atherosclerosis thrombosis

Interleukin-6 (IL-6) is a multifunctional proinflammatory cytokine that is linked to a number of disorders, including multiple myeloma,1 rheumatoid arthritis,2 osteoporosis,3 and Alzheimer’s disease.4 Several recent studies have shown that IL-6 has critical pathophysiological roles in cardiovascular diseases, such as atherosclerosis,5 acute myocardial infarction,6 abdominal aortic aneurysm,7 and congestive heart failure.8 IL-6 mRNA or protein has been detected semiquantitatively and immunohistochemically in animal and human atherosclerotic lesions.9–11 IL-6 is produced by vascular smooth muscle cells (VSMCs) and affects their contraction and proliferation.12,13 Moreover, IL-6 influences other components of atherosclerotic lesions, monocytes/macrophages and endothelial cells, through induction of several factors, such as vascular endothelial growth factor and monocyte chemotactic protein-1,14,15 Thus, detailed characterization of IL-6 production by VSMCs is important to help us understand the pathogenesis of atherosclerosis or postangioplasty restenosis and develop methods for therapeutic modulation of its expression.

Serotonin (5-hydroxytryptamine; 5-HT), a decarboxylated derivative of the amino acid tryptophan, is a naturally occurring vasoactive substance and is a major secretory product of activated platelets. Its receptors have been classified depending on their signal transduction mechanism: 5-HT 1 receptors as adenylyl cyclase inhibitors, 5-HT 2 receptor as a phospholipase C stimulator, and 5-HT 3, 5-HT 4, 5-HT 5 receptors as adenylyl cyclase activators, all of which are members of the G protein–coupled receptor family. The 5-HT 3 receptor subtype is a 5-HT gated channel. In the vascular system, multiple effects of 5-HT are mediated primarily by 5-HT 1 and 5-HT 3 receptors.16 5-HT induces VSMC proliferation, contraction, migration, and platelet aggregation.17–22 5-HT also participates in vascular inflammation associated with atherosclerosis.23 5-HT levels in the coronary sinus are increased significantly in patients with acute coronary syndrome.24 Therefore, 5-HT may play important roles in the progression of local vascular injury associated with atherosclerosis or coronary angioplasty.25,26

Because chronic inflammation of the vessel wall mediated by cytokines is a hallmark of atherosclerosis, the hypothesis tested in this study was that 5-HT may elicit inflammatory signals in VSMCs, the most abundant cells in atherosclerotic...
tissue. We investigated the effects of 5-HT on IL-6 synthesis in cultured human VSMCs.

**Methods**

**Materials**

The selective 5-HT1 receptor antagonist sarpgrelate hydrochloride was a gift from Mitsubishi Tokyo Pharmaceutical Co (Tokyo, Japan). Calphostin C was a kind gift from Kyowa Hakko Kogyo (Tokyo, Japan). 5-HT was purchased from Wako Pure Chemical Industries Ltd. The 5-HT receptor antagonist spiroxatrine, 5HT, receptor agonist 8-hydroxy-2-di-n-propylamino tetralin (8-OH-DPAT) maleate, 5-HT, receptor antagonist ketanserin, and 5-HT receptor agonist α-methyl-5-HT were purchased from Research Biochemicals Inc. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co. All other chemicals used were of the highest grade commercially available.

**Cell Culture**

VSMCs prepared from human aorta were purchased from Clonetics Corp and cultured in smooth muscle basal medium supplemented with 5% heat-inactivated FCS, 5 μg/mL insulin, 2 pg/mL human basic fibroblast growth factor, 50 μg/mL gentamicin, and 50 pg/mL amphotericin B on 10-cm culture dishes or 24-well plates (Falcon). Confluent monolayers of VSMCs (105 cells) between passages 5 and 10 on 24-well plates were washed twice with 500 μL of PBS (0.01 mol/L sodium phosphate, 0.14 mol/L NaCl, pH 7.2), cultured in FCS-free 0.1% BSA-containing media, and used for the experiments.

This investigation was performed according to the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH publication No. 85-23, revised 1985).

**Measurements of IL-6**

IL-6 concentrations in the culture supernatants were determined with an ELISA kit according to the manufacturer’s instructions (Amer sham International). The absorbance at 450 nm was measured, and their concentrations were determined by interpolation of a standard calibration curve. The lower limit of detection of IL-6 was 10 pg/mL.

**IL-6 mRNA Expression**

Confluent VSMCs in 10-cm dishes were used for total RNA extraction with a commercial kit (Nippon Gene Co Ltd) according to the manufacturer’s instructions. The concentrations of human IL-6 mRNA were determined with a Quantikine mRNA colorimetric quantification kit (R&D Systems, Inc) according to the manufacturer’s instructions. Total RNA samples (2 to 5 μg) were hybridized with IL-6 gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes in 96-well microplates. The hybridization solution was then transferred to streptavidin-coated microplates, and the RNA/probe hybrid was captured. After a wash to remove unbound material, anti-digoxigenin alkaline phosphatase conjugate was added. After unbound conjugate had been washed away, substrate solution was added. An amplifier solution was then added, and color developed in proportion to the amount of gene-specific IL-6 mRNA in the original samples. The absorbance at 490 nm was measured, and IL-6 mRNA concentrations were determined by interpolation of a standard calibration curve. The minimum detectable dose of human IL-6 mRNA was 5 amol/mL.

**Gel Mobility Shift Assay**

Nuclear extracts from VSMCs were prepared as follows. After 3 washes in ice-cold PBS, the cells were scraped off the tissue culture dish, resuspended, and sedimented by centrifugation. The cell pellet was lysed in a buffer composed of 20 mmol/L HEPES-KOH (pH 7.9), 0.35 mmol/L NaCl, 20% glycerol, 1% NP-40, 1 mmol/L MgCl2, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 10 μg/mL leupeptin, 0.5 mmol/L DTT, and 0.2 mmol/L PMSF by incubation on ice for 30 minutes. After centrifugation, the supernatant containing the protein fraction was frozen at −80°C. For electrophoretic mobility shift assays, a double-stranded oligonucleotide representing the consensus sequence for nuclear factor-κB (NF-κB) binding (5’TCAACAGAGGACTTTCCGAGGCA-3’) was labeled with [γ-32P]ATP by use of T-4 polynucleotide kinase. Cell proteins (10 μg) and labeled oligonucleotide (50 000 to 70 000 cpm) were incubated for binding of active NF-κB for 20 minutes at room temperature in a buffer containing dI dC, 8% Ficoll 400, 44 mmol/L HEPES-KOH (pH 7.9), 140 mmol/L KCl, 4% glycerol, 0.05% NP-40, 0.1 mmol/L EDTA, 4.4 mmol/L DTT, and 0.06 mmol/L PMSF. Immediately after binding, the protein/DNA complexes were separated from unbound oligonucleotide by electrophoresis in a native 5% polyacrylamide gel in Tris-HCl-EDTA buffer. Autoradiography was performed with the dried gels and Hyperfilm (Amersham). For testing of specificity of NF-κB/DNA binding, antibodies (Santa Cruz Biotechnology) against the p65 subunits of NF-κB were added to the proteins, resulting in further retardation of electrophoretic mobility, or a 160-fold molar excess of unlabeled oligonucleotide was added to the binding reaction, leading to a decrease in NF-κB-bound radioactivity.

**Statistical Analysis**

Data are expressed as mean±SEM of 4 samples, which represented ≥3 separate experiments. Differences were examined by 1-way ANOVA combined with Dunnett’s test, and values of P<0.05 were considered statistically significant.

**Results**

**Effects of 5-HT on IL-6 Production**

VSMCs were cultured in FCS-free medium for 24 hours. As shown in Figure 1A, IL-6 production by VSMCs increased in a time-dependent manner. Addition of 5-HT (10⁻⁸ mol/L) further increased IL-6 production, and after a 24-hour incubation, the levels of IL-6 in the presence of 5-HT were 2-fold higher than those in its absence. Figure 1B shows the concentration-response effect of 5-HT on IL-6 production. 5-HT increased IL-6 production by VSMCs in a dose-dependent manner (10⁻⁹ to 10⁻⁵ mol/L).

**Effects of 5-HT on IL-6 mRNA and NF-κB Expression**

We then investigated the effects of 5-HT on IL-6 mRNA expression in VSMCs. Cells were incubated with various concentrations of 5-HT for 4 hours. As shown in Figure 2, 5-HT dose-dependently (10⁻⁸ to 10⁻⁶ mol/L) increased IL-6 mRNA expression in VSMCs.

Activation of NF-κB is involved in the expression of IL-6 mRNA. Thus, we investigated NF-κB activity by gel mobility shift assay and found that 5-HT activated NF-κB in VSMCs (Figure 3). We confirmed the specificity of the shifted autoradiographic bands in 2 ways: (1) addition of the antibody against the p65 subunit of NF-κB resulted in a further retardation of the mobility of the NF-κB/oligonucleotide complex (“supershift”), and (2) an excess of unlabeled NF-κB consensus oligonucleotides, but not mutated NF-κB oligonucleotides, reduced the signal intensity of the band associated with active NF-κB.

**Effects of 5-HT Receptor Antagonists and Agonists**

In the vascular system, multiple effects of 5-HT are caused primarily by 5-HT1 and 5-HT2 receptors. We thus investigated the effects of their receptor antagonists. As shown in Figure 4, the 5-HT1, receptor antagonist ketanserin and the selective 5-HT2A receptor antagonist sarpgrelate dose-
dependent (10⁻⁸ to 10⁻⁶ mol/L) inhibited the stimulatory effect of 5-HT on IL-6 production. In contrast, the 5-HT₁ receptor antagonist spiroxatrine did not affect the production of IL-6. None of these antagonists showed a significant effect on IL-6 production by 5-HT–unstimulated VSMCs (data not shown).

We then investigated the effects of 5-HT receptor agonists on IL-6 production by VSMCs. The 5-HT₂ receptor agonist α-methyl-5-HT increased IL-6 production by VSMCs in a dose-dependent manner (10⁻⁸ to 10⁻⁵ mol/L), whereas the 5-HT₁ receptor agonist 8-OH-DPAT showed no effect on IL-6 production (Figure 5). These results suggested that the effect of 5-HT is mediated via 5-HT₂A receptors.

**Involvement of Protein Kinase C**

5-HT₁ receptors interact with protein kinase A (PKA), and 5-HT₂ receptors interact with protein kinase C (PKC). We thus investigated the effects of PKA and PKC inhibitors on 5-HT–induced IL-6 production by VSMCs. As shown in Figure 6, the PKC inhibitor calphostin C significantly inhibited IL-6 production induced by 5-HT, whereas the PKA inhibitor KT5720 showed no inhibitory effect.

VSMCs were further exposed to PMA (10⁻⁶ mol/L) for 24 hours to cause functional depletion of PKC activity and then incubated with 5-HT or PMA (10⁻⁷ mol/L) for a further 24 hours. As shown in Figure 7, IL-6 levels increased significantly after addition of 5-HT or PMA in control cells not preincubated with PMA. Conversely, in cells preincubated with PMA for 24 hours, addition of fresh PMA showed no significant effect on IL-6 production, indicating that PKC was functionally inactivated in these cells. 5-HT caused no changes in IL-6 production in these PKC-depleted cells.

**Discussion**

Molecular analysis of 5-HT receptor mRNA expression revealed that 5 of 13 known G protein-coupled 5-HT receptor mRNAs are expressed in blood vessels (5-HT₁b/d, 5-HT₂A, 5-HT₂B, 5-HT₆, and 5-HT₇). Recent studies have shown that 5-HT₂A and 5-HT₁B receptors are expressed predominantly in human coronary arteries and are principally responsible for 5-HT–evoked coronary contraction. 5-HT₂A and 5-HT₁B receptor mRNAs are expressed in cultured human VSMCs. 5-HT₂A receptors located on VSMCs exert strong constrictor activity in blood vessels, 5-HT₂A receptor function is coupled to activation of phospholipase C, resulting in liberation of the second messenger diacylglycerol, which activates PKC, and inositol triphosphate, which mobilizes Ca²⁺ from intracellular stores. Ca²⁺ causes muscle contraction. We have confirmed that 5-HT₂A receptor mRNA is expressed in cultured human VSMCs by RT-PCR (data not shown). Using these cells, we revealed that 5-HT enhances IL-6 synthesis in VSMCs via the 5-HT₂A receptor.

We also showed that the selective PKC inhibitor calphostin C or PKC downregulation by PMA abolished 5-HT–induced IL-6 production by VSMCs. These results indicated that
activation of PKC at least partially mediated the effect of 5-HT on IL-6 synthesis. PKC constitutes a family of proteins including multiple conventional (PMA-activated and Ca\(^{2+}\)-dependent), novel (PMA-activated and Ca\(^{2+}\)-independent), and atypical (Ca\(^{2+}\)-independent and unaffected by PMA or diacylglycerol) isoforms. Conventional (\(\alpha\) and \(\beta\)), novel (\(\epsilon\)), and atypical (\(\zeta\)) isoforms have been detected in VSMCs.\(^{35}\) Previously, we reported that 5-HT activated PKC in cultured VSMCs.\(^{36}\) Although the present study did not address which isoforms of PKC are involved in IL-6 production, 5-HT–induced IL-6 synthesis might be mediated by activation of conventional or novel forms of PKC, because the effect of 5-HT was attenuated by downregulation of PKC. The atypical isoform PKC\(\zeta\) is not induced or downregulated by PMA.\(^{37}\)

We finally demonstrated that 5-HT induces NF-\(\kappa\)B activation in human VSMCs. The promoter regions of the IL-6 gene contain binding sequences for the transcription factor NF-\(\kappa\)B.\(^{38}\) Activated NF-\(\kappa\)B is present in human atheroma,\(^{39}\) and inducible NF-\(\kappa\)B activity is expressed in human VSMCs.\(^{40}\) NF-\(\kappa\)B can be activated through phosphorylation by PKC.\(^{41}\)

Our results indicated that 5-HT–induced IL-6 production is mediated by PKC and NF-\(\kappa\)B activation, which may be a critical pathway for the induction of proinflammatory cytokines in atherosclerotic lesions.

The present study demonstrated that 5-HT acts on the 5-HT\(_{2A}\) receptor and enhances secretion of IL-6, a marker of VSMC inflammation, in human VSMCs. Benedict et al\(^{42}\) reported that plasma concentrations of 5-HT in the coronary sinus were markedly increased in the later stages of coronary

Figure 3. 5-HT activates NF-\(\kappa\)B in human VSMCs. Confluent cells were stimulated with medium alone (lane 1) or 5-HT (10\(^{-6}\) mol/L) (lane 2) for 1 hour. Nuclear proteins were extracted and incubated with \(^{32}\)P-ATP-labeled oligonucleotides corresponding to NF-\(\kappa\)B consensus sequence as described in Methods. Binding of activated NF-\(\kappa\)B to oligonucleotide was visualized by autoradiography after separation by nondenaturing polyacrylamide gel electrophoresis. Solid arrows denote oligonucleotide/NF-\(\kappa\)B protein complex. To test specificity of NF-\(\kappa\)B detection, nuclear extracts were preincubated with a antibody against \(\alpha\)65 subunit (lane 3), and an excess of unlabeled mutant (lane 4) or consensus (lane 5) oligonucleotide was added to reaction mixture. Open arrow, supershift complex.

Figure 4. Inhibitory effects of 5-HT receptor antagonists on 5-HT–induced IL-6 production by human VSMCs. Various concentrations (10\(^{-8}\) to 10\(^{-6}\) mol/L) of 5-HT\(_{1}\) receptor antagonist spiroxatrine (□), 5-HT\(_{2}\) receptor antagonist ketanserin (●), or selective 5-HT\(_{2A}\) receptor antagonist sarpgrelate (○) were added to cells 1 hour before 5-HT treatment (10\(^{-6}\) mol/L) for 24 hours. IL-6 levels were determined by ELISA. Values represent percent inhibition of IL-6 production by antagonists. Data are mean\(\pm\)SEM of 4 samples. *P<0.01 vs control samples not exposed to antagonists.

Figure 5. Dose-dependent effects of 5-HT receptor agonists on IL-6 production by human VSMCs. Cells were incubated for 24 hours with various concentrations (10\(^{-8}\) to 10\(^{-5}\) mol/L) of 5-HT\(_{2}\) receptor agonist \(\alpha\)-methyl-5-HT (●) or 5-HT\(_{1}\) receptor agonist 8-OH-DPAT (○). Data are mean\(\pm\)SEM of 4 samples. *P<0.01 vs control samples not exposed to agonists.
thrombus formation in dogs in vivo. Vikenes et al. reported that plasma 5-HT levels were associated with the presence of coronary artery disease and subsequent cardiac events. In the present study, serotonin at 10^{-7} mol/L or 10^{-5} mol/L significantly increased IL-6 production by VSMCs. It has been reported that plasma 5-HT levels in the coronary sinus blood increase up to 10^{-7} mol/L after PTCA. Thus, 5-HT in the range of in vivo physiological concentrations could increase IL-6 production.

In advanced stages of atherosclerosis, cytokines may promote destabilization and rupture of plaques by induction of matrix-degrading enzymes, ultimately leading to thrombosis and complete obstruction of the vessel. Increased production of IL-6 may be of particular clinical relevance. We previously reported that plasma IL-6 concentrations are raised in patients with acute myocardial infarction and that IL-6 mRNA is expressed in human atherosclerotic lesions. Kaneko et al. observed the expression of IL-6 in the coronary arteries of patients with myocardial infarction. Ridker et al. reported that the plasma level of CRP, which is synthesized in the liver after stimulation with IL-6, in healthy men predicts the risk of future myocardial infarction and stroke. Biasucci et al. reported that increasing levels of serum IL-6 in unstable angina are associated with increased risk of in-hospital coronary events. Recently, we reported that IL-6 levels in the coronary sinus blood became significantly elevated after PTCA and that a positive significant correlation was observed between increased IL-6 levels and late restenosis.

From these findings, we are tempted to speculate that, in addition to its direct effects on vascular smooth muscle contraction and growth, 5-HT secreted from activated platelets may promote the initiation or progression of coronary atherosclerosis by activating IL-6-mediated inflammatory processes in the vascular tissue. However, to prove our premise, further studies are necessary to determine whether 5-HT plays an inflammatory as well as thrombotic role in experimental models or patients with coronary artery disease.

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